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THE SYNTHESIS OF MUCOSA AND LYMPH PHOSPHOLIPIDE DURING FAT ABSORPTION

by

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Running Title:

PHOSPHOLIPIDE AND FAT ABSORPTION

Since Sinclair observed that the phospholipides of the intestinal mucosa incorporate ingested fatty acids during absorption (1) the hypothesis that phospholipides may be intermediate in triglyceride resynthesis has been an attractive one. There has been evidence, however, that although fatty acids are incorporated, and that there is an increased rate of P^{32} turnover in mucosa phospholipides during fat absorption (2, 3) the rate of turnover may not be adequate to account for the amount of fat being absorbed (4).

The present study is an effort to solve this problem by the determination of the relative activity of labeled glycerol and fatty acid in the triglycerides and phospholipides of the mucosa and lymph after the ingestion of triglycerides labeled in both moieties. The results of the study suggest some new concepts on the mechanism of fat absorption.

EXPERIMENTAL

Mucosa Phospholipides: Three experiments were run with three different analytical procedures and two different test fats. In all three, the rats were trained to eat a fat-free ration at 8 A.M. On the morning of the test, 100 mg of the test fat was mixed with 1/2 gm of the fat-free ration. One-half hour later another 1/2 gram of the basal ration was given. The animals were sacrificed three hours after the first meal was offered. They all ate the test meals within a few minutes.

In the first test, some of the conjugated trillinolin previously used in fat absorption studies (5) was used. The entire small intestine, after careful washing and removal of mesenteric fat, was diced and extracted with alcohol-ether. The lipide was reextracted with petroleum ether and the phospholipides precipitated with acetone. The phospholipides were then saponified in 1 N KOH in alcohol and the percentage of conjugated linolenic acid in the recovered acids determined spectrophotometrically (5).

The acidified saponification liquor was oxidized with KIO_4 , the formaldehyde recovered as methylene bismethone and its radioactivity counted as previously described (5).

In the second experiment, tripalmitin, labeled with C^{14} in the α position of the glycerol and in the carboxyl of the acid, was used. The analytical procedures were the same as in the first except that the fatty acid activity was measured by direct counting technique.

Several objections have been raised to the analytical procedure used in the above two experiments. It has been reported (6) that mucosa phospholipide contains considerable quantities of sphingomyelin, which, because of mutual solubility effects, may dissolve in petroleum ether with the rest of the

phospholipide. If this is true, fatty acids would be added to the acids to be counted without the compensating addition of glycerol. A further objection was that the nitrogenous bases from phospholipide saponification, as well as the glycerol phosphate, may produce formaldehyde.

In order to answer these objections and to analyze the mucosa independently of the intestinal musculature, the following procedure was devised and followed:

Six rats were trained and given the test meal as described. The small intestines were removed under ether anesthesia and immediately washed with physiological saline. The mucosa was then squeezed out of the intestine and frozen on dry ice. (To slow autolysis, the intestines were kept on cracked ice when not being handled).

Extraction and purification of lipides: About 6 gms of mucosa were obtained from the six rats. The lipides were extracted and purified according to Johnson et al. (7) except that trichloroacetic acid was washed from the 6:1 petroleum ether-chloroform solution with 0.25 M Mg Cl₂ solution instead of water, according to McKibben (8).

Isolation of Phospholipides: The phospholipides were separated from the combined lipides after the methods of Borgström (9) and Fillerup and Mead (10),

modified as follows:

A column of dry silicic acid, 10 gm per 30 mg of total lipide, was prepared by washing with 3 column volumes of methanol, anhydrous acetone, anhydrous ether and chloroform in turn. The total lipide was dissolved in one column volume of chloroform and put on the column. The phospholipides were adsorbed on the column, washed with chloroform and eluted with methanol. The methanol was removed in vacuo at 55°C in a slow stream of nitrogen. The phospholipide was redissolved in chloroform, transferred to a tared tube, dried under nitrogen and weighed. There were approximately 400 mg.

Saponification: Because of the report that mucosa phospholipides contain large quantities of sphingomyelin (8) the mixed phospholipides were homogenized and differentially saponified by the method of Thannhauser (6) in 0.25 N NaOH at 37°C for 6 days. After decomposition of the soaps at mild acid pH (methyl red), the mixture was chilled in the refrigerator and filtered through a celite pad on a chilled sintered glass filter and washed with water.

Separation of fatty acids of glycerol containing phospholipides: The fatty acids and sphingomyelin plus any other unhydrolyzed phospholipides, were dissolved in chloroform, dried with anhydrous sodium sulfate and filtered. The chloroform

solution, after concentration to one column volume, was placed on a silicic acid column prepared as described above for the separation of phospholipide, and unhydrolyzed phospholipide removed as before. The chloroform solution of fatty acids was concentrated, filtered, dried and weighed. There were 132 mg.

Purification of glycerophosphate: The acidified saponification liquor, after removal of the fatty acids, was freed of ethanolamine and serine by passage through a 10 gm column of Dowex 50 (acid form) in a 0.50 ml burette, prepared by washing with demineralized water.

Preparation of methylene bismethone: The purified glycerophosphate solution was diluted to 50 ml with distilled water, and 50 ml of 4N H_2SO_4 and 36 ml of 0.1 N KIO_4 (equivalent to 0.8 m mol of glycerol) added. The mixture was heated for one hour at 95°-100°C while nitrogen was passed through the solution to flush the resulting formaldehyde into a trap containing 60 ml of 0.4 per cent aqueous dimedone, 40 ml of water, 10 ml of AcONa and acid to pH 4.7 - 5.0.

Preliminary studies have shown that under these conditions choline produces no formaldehyde and that one mol of glycerophosphate produces one mol of formaldehyde.

Mucosa "neutral fat": The chloroform solution, after removal of phospholipides

on silicic acid as described above, was evaporated, and the "fat" saponified with 4 per cent alcoholic KOH. The fatty acids were separated and plated as usual. The acidified saponification liquor was cleared by filtration through Supercel and oxidized with KIO_4 at room temperature and the methylene bismethone prepared in the usual manner.

Determination of radioactivity: The activities, in counts per minute, were determined by direct count on the fatty acids and methylene bismethone.

RESULTS

The percentage of labeled glycerol in the ingested fat, replaced in the recovered lipide by inactive glycerol from endogenous sources, was calculated according to the equation

$$R = 100 \times \frac{A_f}{A_r} \times \frac{G_r}{G_f} \times 100$$

in which R = % replaced glycerol, A_f = activity of fatty acid in the lipide fed, A_r = activity of fatty acid in the recovered lipide, G_f = activity of the glycerol in the lipide fed and G_r = activity of glycerol in the recovered lipide.

The results of the three experiments described above, and the recalculated analyses of lymph triglycerides and phospholipides from previous work (5) are summarized in Table I.

DISCUSSION

The factor "R" defined above as "the percentage of labeled glycerol replaced by inactive glycerol" requires further elucidation. Ingested fatty acids remain unchanged up to the time of their appearance in the lymph except that they are diluted with endogenous fat. They may thus be used as a standard for any changes in glycerol. Thus, if a triglyceride is fed with 100 units of activity in both glycerol and fatty acid, and the lymph triglycerides have 50 units of activity in the fatty acid moiety but only 25 units in the glycerol moiety, there was a 1:1 dilution with endogenous fat. In addition, there was a 50 per cent replacement of labeled exogenous glycerol with inactive endogenous glycerol.

A negative number representing the replacement of ingested labeled glycerol with endogenous glycerol in mucosa "neutral" fat may be explained on the basis that this fraction contained considerable quantities of monoglycerides. This possibility is currently under study.

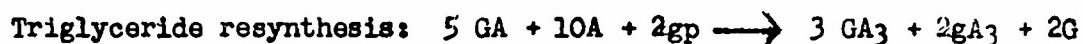
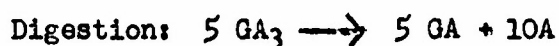
The reasonably close agreement between the values of 58, 62 and 61 per cent replacement of labeled with unlabeled glycerol in the synthesis of mucosa obtained by the use of 3 procedures phospholipide, demonstrates that the precaution of differential saponification to remove sphingomyelin from the total lipide, and the ion exchange procedure to

remove serine and ethanolamine from the acidified saponification liquor, are unnecessary. Evidently the reextraction of the alcohol-ether extract with petroleum ether is adequate for eliminating the sphingomyelin and, under the conditions used, (acid pH and room temperature for one hour), serine and ethanolamine are not oxidized to formaldehyde with KIO_4 . Other studies, with the pure bases, as well as choline, have also shown that, under these conditions, formaldehyde is not a product of their oxidation with KIO_4 .

The reasonably close agreement between mucosa and lymph phospholipides indicate that the two have the same origin.

The replacement of 60 per cent of the labeled glycerol in ingested fat to form mucosa and lymph phospholipide, but only 40 per cent to form lymph triglyceride, suggests that triglyceride is the precursor of phospholipide rather than the converse.

These observations may be interpreted by the following scheme in which G represents labeled glycerol, A labeled fatty acid, gp unlabeled endogenous glycerol or its precursor, and PB a phosphorylated base.





According to this hypothesis monoglycerides are the end product of fat digestion. The absorbed monoglycerides and glycerol, or its precursor (possibly dihydroxyacetone), compete in the mucosa for the available fatty acids, the monoglycerides forming three mols of triglyceride to the glycerol's two. There is thus a loss of 40% of the original glycerol. Some of the resynthesized triglyceride then forms mucosa and lymph phospholipide.

The above hypothesis has the attractive feature that it not only can explain the degree of replacement of labeled with unlabeled glycerol reported here, but it also resolves most of the past controversies.

Recent studies in enzymatic digestion of fat show very convincingly that the first molecule of fatty acid is quite easily hydrolyzed, the second is removed with somewhat more difficulty, but the last requires special conditions for hydrolysis (11, 12, 13, 14, 15). These observations suggest that the loss of 40 per cent of the glycerol of ingested fat may not take place in the lumen of the intestine during digestion. This concept is strengthened by the observations that ingested monoglycerides are hydrolyzed 70 per cent, just a little more than enough to form triglycerides (16), and ingested triglycerides are hydrolyzed 40 per cent (5). The regularity of these changes suggest an intracellular

rather than an extracellular (digestion) mechanism.

The concept that monoglycerides are the end product of fat digestion also satisfies the convincing evidence that complete hydrolysis need not occur and the fact that considerable hydrolysis does occur.

Ingested free fatty acids are always found in the lymph as triglycerides.

Ingested monoglycerides are also found in the lymph as triglycerides (16).

When both monoglycerides and free fatty acids are being absorbed, it is, therefore, reasonable to expect competition between the monoglyceride and endogenous glycerol, or its precursor, for the available fatty acids. This would result in the substitution of unlabeled glycerol for labeled glycerol, as indicated.

The postulate that not glycerol, but a precursor, possibly dihydroxyacetone, is involved in triglyceride and phospholipide resynthesis explains the failure of numerous observers to note the incorporation of more than traces of labeled free glycerol into glycerides when fed with fat or free fatty acids (17, 18, 19, 20, 21). Only that fraction of ingested glycerol which forms the ketone derivative would be available for glyceride synthesis. The concept also explains the failure of glycerol, hydrolyzed from fat during digestion and absorption, to be reutilized (5, 19, 20).

The formation of phospholipide as a product of resynthesized triglycerides resolves the conflicting observation which clearly demonstrates the incorporation of ingested fatty acids (1) and the rate of turnover studies with P^{32} which indicate that phospholipides cannot be intermediate in triglyceride synthesis (4).

Finally, the apparent contradictions in the observations that intralumenary lipolysis proceeds only to monoglyceride stage, and the clear cut evidence that glycerol is lost and not reused during absorption, is resolved.

At least one other possibility has not been eliminated. In the present work the lecithins, cephalins and phosphatidic acids, if present, were analyzed together. It is possible that only one contained the active glycerol, and the fatty acids were distributed between two or all three. The activity of the glycerol in the involved phosphorylated derivative could then be the same as in the triglycerides and the phospholipide interpreted as being intermediate². There would be no dilution or replacement of glycerol in the formation of triglycerides from phospholipide.

CONCLUSIONS

1. Triglycerides, resynthesized from absorbed monoglycerides, fatty acids and endogenous glycerol, or its precursor, are the precursors of mucosa and lymph

phospholipides.

2. The following concepts were developed to account for the observed facts and previous studies:

- a. Monoglycerides are the end product of fat digestion in the lumen of intestine.
- b. The absorbed monoglycerides and endogenous glycerol, or its precursor, compete in the ratio of 3 to 2 for the absorbing fatty acids to form triglycerides.

Table I. Percentage replacement of labeled with unlabeled glycerol in intestinal mucosa and lymph lipides after the ingestion of C^{14} labeled glycerol as tripalmitin and as conjugated trillinolin.

Triglycerides		Phospholipides	
Mucosa	Lymph	Mucosa	Lymph
-54 ⁴	43 ¹	58 ²	52 ¹
	46 ¹	62 ³	54 ¹
	34 ¹	61 ⁴	
	27 ¹		
	40 ¹		

¹ Recalculated from previous work (5).

² From conjugated trillinolin experiment herein reported.

³ Obtained from labeled tripalmitin experiment with single analytical procedure, herein reported.

⁴ Obtained from labeled tripalmitin experiment with chromatographic separation of phospholipide, differential saponification and chromatographic separation of nitrogenous bases, herein reported.

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Notes

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2. In a previous report from this laboratory (Federation Proceedings 12, 257 (1953)

the compliment of the percentage of glycerol replaced in mucosa phospholipides

was mistaken for the percentage replaced. Since the latter was 58, the error

made it appear to be 42, the same as found for lymph triglycerides. This

error led to the faulty conclusion that phospholipides are intermediate.